Concise report

Novel carbonic anhydrase autoantibodies and renal manifestations in patients with primary Sjögren’s syndrome

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Abstract

Objective. Anti-carbonic anhydrase II (anti-CA II) antibodies have been related to renal manifestations of primary SS (pSS), and animal studies have even suggested a pathogenic role for them. However, not all pSS patients with renal tubular acidosis (RTA) present with anti-CA II antibodies. Recently, several novel CA isoenzymes have been recognized and we aimed to investigate whether antibodies to these are associated with renal manifestations of pSS.

Methods. We examined anti-CA II antibodies as well as anti-CA I, VI, VII and XIII antibodies by ELISA tests in 74 pSS patients on whom detailed nephrological examinations had been performed and, as controls, in 56 subjects with sicca symptoms, but no pSS.

Results. The levels of anti-CA I, II, VI and VII antibodies were significantly higher in patients with pSS compared with subjects with sicca symptoms but no pSS. None of the anti-CA antibodies was associated with the presence of complete or incomplete RTA or proteinuria or urinary amino acid excretion in patients with pSS. However, levels of anti-CA II, VI and XIII antibodies correlated significantly with urinary pH, and inversely with serum sodium concentrations. The degree of 24-h urinary protein excretion correlated weakly with levels of anti-CA VII antibodies.

Conclusion. Not only antibodies to CA II, but also anti-CA VI and XIII antibodies seem to be associated with renal acidification capacity in patients with pSS.

Key words: Autoantibodies, Carbonic anhydrase, Renal tubular acidosis, Sjögren’s syndrome, Urinary pH.

Introduction

Primary SS (pSS) is a chronic rheumatic disease characterized by dry eyes and mouth, possible extraglandular symptoms and abundant autoantibody production as a sign of autoimmunity [1]. Subclinical or clinical renal manifestations belong to the clinical spectrum of the syndrome [2]. The most typical renal histological manifestation in pSS is interstitial nephritis [3], which may present clinically as distal or proximal renal tubular acidosis (dRTA or pRTA), nephrocalcinosis, nephrogenic diabetes insipidus or Fanconi syndrome [4]. We have previously investigated the occurrence of renal manifestations in patients with pSS and observed that latent or overt dRTA occurred in up to 33%, and mild proteinuria (>0.15 g/24 h) in 44% of the patients [5].

A defect in hydrogen ion secretion is the major pathophysiological mechanism leading to dRTA [6]. In overt dRTA metabolic acidosis is found, while the urine is alkaline. In patients with latent dRTA, inability to acidify the urine is observed only during induced acidosis, i.e. by an oral ammonium chloride loading test.

Carbonic anhydrases (CAs) are key enzymes in the regulation of acid-base balance in both physiological and pathological states [7]. They catalyse the reversible hydration of carbonic dioxide according to the reaction: 

\[ \text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+ \].

An autosomally recessively
inherited shortage of the CA II enzyme has been described as being associated with osteopetrosis, RTA and calcification of the brain [8]. Autoantibodies to CA II have been observed in the sera of patients with SS and SLE, as well as in the distal tubules of the kidney [9]. In addition to the fact that genetic defects of the CA II enzyme have been found to cause RTA, also induction of anti-CA II antibodies has recently been observed to cause RTA in a mouse model of SS [10]. Lymphocytic and plasma cell infiltration was seen in the kidneys of CA II-immunized mice but not in the controls [10].

Recently, several new CA isoenzymes have been discovered [7]. The CA enzyme family is currently known to include a total of 13 active isoenzymes in mammals. As anti-CA II antibodies have been suggested to be involved in the pathogenesis of renal manifestations of pSS, we here sought to establish whether antibodies to other CAs, in particular to the most novel antigen, CA XIII, are related to renal manifestations of pSS. To this end, we analysed autoantibodies to CA I, II, VI, VII and XIII in 74 patients with pSS, on whom detailed renal examinations had been performed [5].

**Subjects and methods**

**Subjects**

Serum samples were obtained after informed consent from 74 patients with pSS (72 females and 2 males) and from 56 subjects presenting with sicca symptoms (46 females and 10 males), but not fulfilling the criteria for pSS. The data collections from the pSS patients and controls have been described in detail elsewhere [5, 11]. The mean (s.o.) age of the patients with pSS was 58 (12) years and range 29–82 years, and the mean duration of their disease was 9 (4) years. The mean age of the control subjects with sicca symptoms was 55 (13) years and range 28–80 years. A careful clinical examination together with an in-depth interview with the patients covering previous and concurrent diseases and medications and duration of pSS had been conducted [5].

**Expression and purification of CAs**

Human CA I and CA XIII enzymes were expressed in *Escherichia coli* as glutathione S-transferase-tagged protein and then affinity purified on a glutathione Sepharose column (GE Healthcare, Buckinghamshire, UK) according to the manufacturer’s protocol. Production and characterization of human CA VII recombinant enzyme has recently been described by Bootorabi *et al.* [12]. The recombinant human CA II enzyme was produced in *E. coli* and purified using CA inhibitor affinity chromatography as described earlier [13]. The affinity chromatography matrix, *p*-aminomethylbenzenesulfonamide–agarose, was purchased from Sigma-Aldrich (St Louis, MO, USA). Secretory CA VI enzyme was isolated from human milk according to the published protocol [14].

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**Standard laboratory tests**

The standard laboratory tests included serum creatinine, sodium and potassium and urinalysis. A 24-h urine collection was gathered and creatinine clearance and excretion of urinary total protein determined; urinary excretion of albumin, immunoglobulin G (IgG) and α1-m was determined from a sample obtained in timed overnight urine collection by methods described elsewhere [5]. An ammonium chloride loading test had been performed in 51 of these patients, as previously described [5].

**Detection of autoantibodies against CA enzymes**

Autoantibody levels were determined using an ELISA developed in our laboratory. The protocol for the immunoassay included the following steps: purified CA I, II, VI, VII and XIII proteins (50 ng/well) in sodium carbonate buffer (15 mM Na2CO3, 30 mM NaHCO3, pH 9.6) were incubated on 96-well plates (Thermo Scientific Pierce, Waltham, MA, USA) overnight at 4°C. The wells were washed three times with PBS containing 0.05% Tween-20 (PBST) and blocked with 2% BSA/PBST for 2 h at room temperature. After washing three times with PBST, serum samples were diluted (1:5000) in 2% BSA/PBST and incubated (50 μl/well) overnight at 4°C. Each sample was analysed as triplicate. After washing three times with PBST, diluted (1:25 000) peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was added (50 μl/well) and incubated for 1 h at room temperature. The plates were washed three times with PBST and incubated with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Thermo Scientific Pierce) for 15 min. Reaction was stopped with 2 M H2SO4 and absorbance measured at 450 nm (Fig. 1). Serum samples from two healthy subjects (male and female) served as controls to estimate the baseline level of absorbance. The relative autoantibody levels were determined by dividing the mean absorbance levels of each sample (measured in triplicate) by the mean absorbance of the control samples.

**Statistical methods**

Anti-CA antibody levels were handled as continuous variables, and Mann–Whitney U-test was used for comparisons between the groups. Correlations were calculated with the Spearman’s correlation coefficient. Findings were considered statistically significant at *P* < 0.05. Statistical analyses were performed with SPSS 15.0 for Windows.

**Ethical approval**

The study was approved by the Ethics Committee of Tampere University Hospital, Tampere, Finland.

**Results**

The levels of anti-CA I, II, VI and VII antibodies differed significantly between patients with pSS and the control subjects suffering from sicca symptoms but not fulfilling the criteria for pSS (Fig. 2). Of the 74 patients with pSS, 33 (45%) had mild proteinuria (defined as total urinary...
protein excretion $\geq 0.15$ g/24 h; range 0.15–0.42 g/24 h) and 16 (31%) out of 51 subjects for whom ammonium chloride loading tests had been performed had overt or latent dRTA [5].

There were no significant differences between pSS patients with overt or latent dRTA ($n = 16$) and those with no dRTA ($n = 35$), in the median [interquartile range (IQR)] levels of anti-CA I antibodies [1.24 (1.11–1.41) vs 1.33 (1.25–1.49), $P = 0.123$], anti-CA II antibodies [2.12 (1.54–2.86) vs 2.12 (1.84–3.06), $P = 0.730$], anti-CA VI antibodies [1.50 (1.31–1.66) vs 1.65 (1.32–1.94), $P = 0.174$], anti-CA VII antibodies [1.20 (1.12–1.29) vs 1.25 (1.13–1.37), $P = 0.247$] or anti-CA XIII antibodies [1.47 (1.30–1.62) vs 1.63 (1.35–1.86), $P = 0.150$].

None of the studied anti-CA antibodies (anti-CA I, II, VI, VII and XIII antibodies) was associated with the presence of proteinuria (data not shown) in these patients with pSS, nor were they associated with the presence of tubular proteinuria as judged by the degree of urinary excretion of $\alpha_1$-m (Table 1). However, urinary pH was significantly associated with the levels of anti-CA II, VI and XIII antibodies, and the levels of these antibodies correlated inversely with serum sodium levels (Table 1). Moreover, the degree of 24-h urinary protein excretion correlated with levels of anti-CA VII antibodies and there was a non-significant trend towards a correlation between anti-CA VI antibodies and urinary IgG excretion (Table 1).

There was slight cross-reactivity between the different CA isoenzymes, i.e. nearly all of the antibodies against the different CA isoenzymes had at least weakly positive correlations between each other (data not shown). In particular, there was a significant correlation between anti-CA I and VII antibodies ($r = 0.838$, $P < 0.0001$) and between anti-CA VI and XIII antibodies ($r = 0.873$, $P < 0.0001$).

Discussion

The main finding in the current study was that in addition with the classical anti-CA II antibodies also anti-CA VI and XIII antibodies correlated significantly with urinary pH in patients with pSS and they might therefore be associated with renal acidification capacity in these patients. In addition to their association with urinary pH, anti-CA II, VI and XIII antibodies all correlated inversely with serum sodium levels. Taken together, these findings might imply the
Table 1: Correlation ($r$) of serum anti-CA enzyme antibody (anti-CA-ab I, II, VI, VII and XIII) levels with laboratory findings in 74 patients with pSS (Spearman’s correlation coefficient)

<table>
<thead>
<tr>
<th>Variable</th>
<th>CA I</th>
<th>$P$</th>
<th>CA II</th>
<th>$P$</th>
<th>CA VI</th>
<th>$P$</th>
<th>CA VII</th>
<th>$P$</th>
<th>CA XIII</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum sodium</td>
<td>0.006</td>
<td>0.963</td>
<td>-0.328</td>
<td>0.004</td>
<td>-0.346</td>
<td>0.003</td>
<td>0.027</td>
<td>0.818</td>
<td>-0.325</td>
<td>0.005</td>
</tr>
<tr>
<td>Serum potassium</td>
<td>0.083</td>
<td>0.482</td>
<td>-0.222</td>
<td>0.854</td>
<td>-0.101</td>
<td>0.39</td>
<td>0.253</td>
<td>0.03</td>
<td>0.014</td>
<td>0.906</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>-0.001</td>
<td>0.991</td>
<td>0</td>
<td>0.999</td>
<td>0.016</td>
<td>0.895</td>
<td>0.06</td>
<td>0.609</td>
<td>0.08</td>
<td>0.496</td>
</tr>
<tr>
<td>U-pH</td>
<td>0.01</td>
<td>0.935</td>
<td>0.25</td>
<td>0.032</td>
<td>0.28</td>
<td>0.016</td>
<td>0.029</td>
<td>0.803</td>
<td>0.322</td>
<td>0.005</td>
</tr>
<tr>
<td>24-hour urinary protein excretion</td>
<td>0.135</td>
<td>0.25</td>
<td>0.014</td>
<td>0.908</td>
<td>0.032</td>
<td>0.785</td>
<td>0.253</td>
<td>0.03</td>
<td>0.071</td>
<td>0.55</td>
</tr>
<tr>
<td>Creatinine clearance</td>
<td>-0.188</td>
<td>0.109</td>
<td>0.019</td>
<td>0.872</td>
<td>0.161</td>
<td>0.172</td>
<td>-0.216</td>
<td>0.064</td>
<td>0.074</td>
<td>0.531</td>
</tr>
<tr>
<td>cU-alb</td>
<td>0.059</td>
<td>0.616</td>
<td>0.023</td>
<td>0.843</td>
<td>0.166</td>
<td>0.158</td>
<td>0.074</td>
<td>0.53</td>
<td>0.145</td>
<td>0.216</td>
</tr>
<tr>
<td>cU-IgG</td>
<td>0.004</td>
<td>0.97</td>
<td>0.077</td>
<td>0.513</td>
<td>0.226</td>
<td>0.053</td>
<td>0.03</td>
<td>0.801</td>
<td>0.141</td>
<td>0.231</td>
</tr>
<tr>
<td>cU-21r,m</td>
<td>0.021</td>
<td>0.86</td>
<td>0.062</td>
<td>0.601</td>
<td>0.151</td>
<td>0.199</td>
<td>0.1</td>
<td>0.397</td>
<td>0.125</td>
<td>0.287</td>
</tr>
</tbody>
</table>

cU collected urine: timed overnight collection.

The significance of all of these antibodies in the regulation of acid-base homeostasis in patients with pSS.

In previous studies, anti-CA II antibodies have been found to be associated with SS [9, 15] and among patients with SS, those with dRTA had higher levels of anti-CA II antibodies than those without RTA [15]. Findings in these previous studies indicate that dRTA in SS may be caused, at least in some patients, by defective CA II function resulting from high plasma levels of anti-CA II autoantibodies. Our results showing anti-CA II antibodies to correlate with urinary pH are thus in line with these previous reports.

However, not all patients with pSS and RTA have been positive for anti-CA II antibodies [15]. As anti-CA II antibodies are detected in only a portion of patients with SS and RTA, it is unlikely that CA II is the only antigen recognized in pSS patients with renal involvement. The cytoplasmic CA XIII is the most recently recognized human CA isoenzyme, also shown to be expressed in the kidney [7]. In human tissues, CA XIII gene expression has been identified in both renal cortex and medulla, strongest immunoreactions being localized to the collecting ducts, similarly to CA II [16]. In view of its localization, it is not surprising that antibodies to CA XIII seemed to be associated with urinary acidification capacity in our pSS patients. CA XIII has been assumed to have a role in the maintenance of the acid-base balance in the kidney [7], and our results lend support to this conception. This is, in fact, the first time that antibodies to this novel CA XIII isoenzyme have been found related to urinary acidification. There was, however, considerable variation in the levels of CA XIII autoantibodies.

CA VI appeared to represent a third isoenzyme targeted by autoantibodies correlating with urinary pH. This finding was surprising in that CA VI protein has never been demonstrated in the kidney. CA VI is the only secretory CA isoenzyme and is found particularly in the saliva and known to be expressed in the acinar cells of the parotid and submandibular glands [17], i.e. in organs typically affected in pSS. It remains unclear, however, how the CA VI autoantibodies can contribute to urinary pH regulation. Anti-CA VI antibodies correlated strongly with anti-CA XIII antibodies, so cross-reactivity with antibodies against the CA XIII expressed in the kidneys is not excluded as a possible explanation.

In addition to their association with urinary pH, anti-CA II, VI and XIII antibodies correlated inversely with serum sodium. This is fully plausible, since exchange of sodium with hydrogen ions occurs in the proximal tubulus, and if hydrogen ion secretion is deficient, sodium exchange cannot take place, resulting in increased excretion of sodium to the urine and hyponatraemia. The most common electrolyte disturbance observed in patients with pSS and dRTA is hypokalaemia [18], but hyponatraemia is also possible [19], and indeed, sodium bicarbonate is used as a therapy for dRTA [20].

Levels of serum anti-CA VII antibodies correlated significantly with serum potassium concentrations. Usually, overt dRTA is associated with hypokalaemia, and in severe cases, albeit infrequently, with hypokalaemic periodic paralysis [18], making the observed positive correlation of anti-CA VII antibodies with serum potassium concentrations unexpected. As stated above, the levels of anti-CA VII autoantibodies were also weakly associated with urinary protein excretion.

In spite of the direct association of anti-CA II, VI and XIII antibodies with urinary pH in our pSS patients, none of the anti-CA antibodies studied was, in fact, associated with latent dRTA or proteinuria. Previously, only anti-CA II antibodies have been studied in pSS and RTA [10, 14], but our results here differ from these previous studies in that we did not observe an association of anti-CA II antibodies with latent or overt dRTA in patients with pSS.

In conclusion, our findings suggest that antibodies to CAs might be involved in renal acidification capacity in patients with pSS. The autoantibody response to the novel anti-CA XIII enzyme is especially interesting, as this isoenzyme is expressed in the kidney. Also the anti-CA VI antibodies might be involved in the process. Further studies regarding the role of these new anti-CA
antibodies are warranted to clarify the mechanisms underlying the development of dRTA in patients with pSS and to resolve whether these novel autoantibodies impair the function of the corresponding isoenzymes.

**Rheumatology key messages**

- Levels of autoantibodies to CA isoenzymes correlate with urinary pH in SS.
- Levels of CA autoantibodies correlate inversely with serum sodium concentrations in SS.

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**References**